DNA methylation modulates *Salmonella enterica* serovar Typhimurium virulence in *Caenorhabditis elegans*

Javin P. Oza, Jimmy B. Yeh, Norbert O. Reich *

Department of Chemistry and Biochemistry, University of California, Santa Barbara, CA 93106, USA

Received 20 August 2004; received in revised form 15 February 2005; accepted 16 February 2005

First published online 18 March 2005

Edited by R.Y.C. Lo

Abstract

*Salmonella enterica* serovar Typhimurium was previously shown to be virulent in *Caenorhabditis elegans*. Here we demonstrate that DNA adenine methyltransferase (DAM) modulates *Salmonella* virulence in the nematode, as it does in mice. After 5 days of continual exposure to bacteria, twice as many worms died when exposed to the wild-type than the dam-mutant strain of *Salmonella*. Similar trends in virulence were observed when worms were exposed to *Salmonella* strains for 5 h and transferred to the avirulent *Escherichia coli* OP50. While a 10-fold attenuation was observed in the absence of DAM, the dam-strain was still able to infect and persist in the host worm. Our results further support the use of *C. elegans* as an accessible and readily studied animal model of bacterial pathogenesis. However, our results suggest that crucial host responses differ between the murine and nematode models. Additionally, we carried out preliminary liquid culture based experiments with the long term goal of developing high throughput animal based screens of DAM inhibitors.

© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: DNA methylation; Virulence; *Caenorhabditis elegans*; *Salmonella typhimurium*

1. Introduction

Studies of the free-living nematode *Caenorhabditis elegans* have provided important insights with relevance to mammals [1–3]. However, *C. elegans* has only recently been studied as a model organism in mammalian host–pathogen interactions. These studies have provided insights into the virulence mechanisms of the pathogenic organisms and have established an accessible model for studying host responses. Ausubel and coworkers [4] first used the nematode worm in solid media based assays to study host-pathogen interactions with human bacterial pathogens *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium [5,6], and with the human fungal pathogen *Cryptococcus neoformans* [7]. These studies demonstrated that mammals and *C. elegans* share similar infection-like mechanisms. In addition, liquid media studies using *Streptococcus pyogenes* [8] and *Bacillus thuringiensis* [9] showed that the release of diffusible exotoxins provides a means to express virulence.

Methylation of DNA at the N6-adenine position, which is not found in mammals, is a primary example of an epigenetic change wherein gene expression is altered without DNA sequence modification [10,11]. Methyltransferases involve an unusual mechanism in which the target base is stabilized outside the B-form double helix during catalysis [12]. The product, N6-methyladenosine, alters the DNA structure and affects DNA-protein interactions. Consequently, DNA
Adenine Methyltransferases (DAMs) regulate DNA replication, methyl-directed mismatch repair, transcription, viability, and virulence in various bacteria [13]. The majority of prokaryotic DNA methyltransferases have a cognate restriction endonuclease, but a small set of MTases lack such a partner. DAM is one such enzyme, and is prevalent among enteric bacteria, including *Escherichia coli*, *Salmonella* species, *Yersinia* species, and *Vibrio cholerae*. In uropathogenic *E. coli*, DAM controls the expression of Type I pili that are required for adherence to host cells [14]. *Salmonella* DAM mutants displayed defects in protein secretion, cell invasion, and M cell cytotoxicity [15], such mutants were also unable to colonize deep tissue in a murine model and were avirulent [16]. Although DAM is not essential for viability in *E. coli* and *Salmonella*, it is required for the growth and viability of both *V. cholerae* and *Yersinia pseudotuberculosis* [17]. Since mammals exploit DNA cytosine methylation and lack any detectable DNA adenine methylation, the bacterial enzymes have become the target of antibiotic development [18].

Our objectives were to validate the use of *C. elegans* to study mammalian pathogens by demonstrating that *S. enterica* serovar Typhimurium DAM is important for pathogenesis in the nematode as demonstrated in mice, and to use this information to determine which virulence genes are regulated by DAM. Further, a major factor in drug discovery efforts is the transition from in vitro, protein and cell-based screens to those involving animal studies. Recent advances in using isogenic cell lines [19], gene array-based expression analysis [20], and various multi-mode fluorescence-based detection approaches are encouraging [21]. However, an animal model that provides an intermediate level of compound screening prior to cost- and time-intensive studies using mammals would be attractive. In particular, an animal model that provides information about lead compound efficacy and toxicity would be particularly useful. *C. elegans* provides many attractive features in this context. The molecular architecture of vertebrate and invertebrate systems, including drug metabolism and nervous systems, is mostly shared [22]. It is estimated that ~0.6% of the 16,000 known *C. elegans* genes code for cytochrome P450 enzymes, and the vast majority are closely associated to the three families of mammalian drug metabolizing enzymes [23]. While clearly not a substitute for mammalian toxicity studies, recent efforts to study both toxicity [24,25] and metabolism [22] in *C. elegans* provide support for its use as an initial, rapid and inexpensive screen.

Our experiments were designed largely to determine whether DAM modulates *Salmonella* virulence in *C. elegans*. This would further validate *C. elegans* for pathogenesis studies and provide a basis for screening DAM inhibitors. We adapted the solid media killing assay used by Ausubel [5], and a liquid assay detection procedure based on a prior method developed by Pomeroy et al. [26] (Union Biometrica).

### 2. Materials and methods

#### 2.1 Strains and media

*E. coli* OP50, *S. enterica* serovar Typhimurium SL1344 (wild-type), and *S. enterica* serovar Typhimurium SV4392 (dam-) [15] were grown at 37 °C overnight in Luria-Bertani broth. Bacterial lawns used for killing experiments were prepared using modified nematode growth media (NGM) agar (0.35% peptone) seeded with 10 μl of bacteria on large 100 mm plates. Plates were incubated at 37 °C for 24 h and allowed to equilibrate to room temperature before seeding worms onto the lawns. N2 and JK509 strains of *C. elegans* were maintained in a 15 °C incubator as hermaphrodites growing on NGM while feeding on *E. coli* OP50. JK509 worms were incubated at 25 °C during experiments for sterility [27].

#### 2.2 Plate-based worm killing assay

*C. elegans* N2 worms were synchronized to the L4 stage while feeding on *E. coli* OP50. NGM experimental plates (10 mm) were prepared by spreading 10 μl of bacteria into a lawn and incubating at 37 °C for 24 h. Ten worms were picked onto each plate using a metal wire loop. Each experiment was carried out in triplicate. Worms were transferred every 24 h to fresh lawns and were scored as dead when failing to respond to physical stimulus [5]. Worms that died by adhering to the Petri-plate walls were not included in the analysis. Three experiments were carried out at 25 °C, each one altering the amount of virulent bacteria being fed to the nematodes. Time required for 50% of worms to die (TD50) and the associated standard error were determined by extrapolation.

#### 2.3 Worm shift killing assay

Synchronized L4-stage N2 *C. elegans* worms (10) were fed SL1344 wild-type *Salmonella* for 5 h on a 100-mm NGM plate [5]. In the same manner, another 10 synchronized L4-stage worms were fed on a 100 mm NGM plate with SV4392 dam-mutant lawn for 5 h. Both experimental groups were then transferred onto *E. coli* OP50 seeded 10 mm plates and transferred every 24 h to fresh OP50 lawns. Control groups were fed on OP50 10 mm plates. Each experiment was carried out in triplicate. Worms were scored as dead when physical stimuli failed to generate any response. Worms that died by adhering to the Petri-plate walls were discounted.
2.4. Bacterial dilution assay

Overnight cultures of wild-type and dam-Salmonella in Luria-Bertani broth were diluted 1:1000 and 1:100 with E. coli OP50 cultures [5]. Ten μl of each of the dilutions were seeded to 10 mm NGM plates and spread into lawns. Lawns were incubated at 37 °C for 24 h and were allowed to equilibrate to room temperature before seeding with 10 N2 C. elegans. Worms were transferred daily to new Salmonella: OP50 lawns and dead worms were scored when physical stimulus generated no movement. The same experiment was done with JK509 worms. Worms that died by adhering to the Petri-plate walls were discounted.

2.5. Liquid killing fluorescence calibration assay

One 10-mm stock plate of live synchronized L4 stage JK509 worms was washed with M9 media with streptomycin (100 μg ml⁻¹) and suspended into 4 ml of complete S. basal medium. A 2-ml aliquot was placed into a 15-ml falcon tube and heat-killed in a 50 °C water bath for 15 min. Both samples of worms were stained with SYTOX green nucleic acid stain (Molecular Probes), a fluorescent green dye that penetrates cells with compromised plasma membranes, but will not cross the membrane of live cells. Dead and live stained worms were titrated into seven dead:live worm ratio groups (60 worms in each 60 μl aliquot), added to 56 wells of a 96-well micro-titer plate (Costar, Corning, NY USA), giving eight replicates for each of the seven groups. The number of worms in each well was standardized to 60 worms by counting the number of worms in 10 μl aliquots of a well-mixed worm suspension under a microscope and diluting or concentrating the sample accordingly. Fluorescence measurements were done using a Perkin–Elmer HTS-7000 Bio Assay Reader with excitation filters set at 485 nm and emission measured at 595 nm [26].

2.6. Liquid killing assay

JK509 mutant C. elegans worms were prepared by washing three 10 mm stock NGM plates of synchronized L4 worms into M9 with streptomycin twice. A final wash was done using 9 ml of Complete S. Basal Medium. Worms were incubated in a 26 °C water bath for 30 min to prevent the development of viable progeny.

Overnight bacterial cultures were centrifuged at 14,000 rpm for 10 min and the pellet was suspended into 1 ml of complete S. basal medium. Each 1 ml of bacterial suspension was added to 3 ml of worms to give a total experimental culture of 4 ml. Worm cultures were rocked overnight at 70 rpm (Falcon Tubes, 15 ml). The liquid medium was changed daily to prevent dauer development.

The suspended worms (1 ml) from each culture were removed daily and treated with SYTOX Green nucleic acid stain. All liquid assays were done in 96-well micro-titer cell-culture plates. Aliquots of 60 μl with 60 worms were assayed in replicates of 8. Fluorescent measurements were done using the Perkin–Elmer HTS-7000 Bio Assay Reader with excitation filters set at 485 nm and emission set at 595 nm [26].

3. Results and discussion

3.1. Salmonella dam-mutant shows attenuated virulence in C. elegans

A general killing assay, where the worms were exposed to 100% Salmonella lawns, evaluated the general killing ability of the dam-mutant strain (SV4392). Over the course of 7 days, 90% of the worms fed on wild-type Salmonella died with the time required for 50% of worms to die (TD₅₀) of 4.5 ± 0.3 days. The dam-mutant strain exhibited an attenuated killing ability, as only 60% of worms had died after 7 days (TD₅₀ = 6.5 ± 0.3 days). Though this partial attenuation is statistically significant, the mutant is not avirulent since less than 20% of worms fed E. coli OP50 had died after 7 days, as expected [5] (Fig. 1).

An alternative assay developed by Ausubel and coworkers was used to test the ability of Salmonella to infect and proliferate in the C. elegans host after a brief exposure [5]. Worms were exposed to the wild-type and dam-mutant Salmonella strains for 5 h and moved to

![Fig. 1. Death rate of C. elegans in N2 on NMM plates, feeding on E. coli OP50, WT Salmonella SL1344, and dam-Salmonella SV4392 over a 1-week period. Worms fed on WT Salmonella (triangles) exhibited higher levels of killing than worms feeding on dam-Salmonella (squares). The control worms were fed E. coli OP50 (circles). Values shown are means of ±standard error of triplicates.](https://example.com/figure1.png)
100% OP50 lawns. After 5 days, 45% of worms fed wild-type Salmonella and 25% of worms fed the dam-strain had died with TD<sub>50</sub> values of 5.4 ± 0.4 and 7 ± 0.5 days, respectively (Fig. 2). Thus, the ability of the Salmonella mutant to infect and persist in the host C. elegans is also attenuated with statistical significance under these conditions.

3.2. S. enterica serovar Typhimurium exhibits killing in C. elegans mutant strain JK509

The purpose of investigating liquid media conditions was to provide a basis for characterizing large numbers of DAM inhibitors in relatively small volumes to minimize material cost and consumption. The transition from solid to liquid media posed significant challenges. One factor is worm fertility and the exponentially increasing population of worms. In solid media, this was solved by separating the experimental nematodes from their progeny through the daily transfer to fresh bacterial lawns. In liquid culture, however, the production of progeny and the inability to remove them made it difficult to quantify the number of live experimental worms. Thus, we exploited the conditionally sterile strain (JK509) with a deletion in the glp-1 gene [27]. The JK509 strain is fertile at 15°C but not at 25°C.

We first tested the JK509 worms in a solid media assay with 1:100 and 1:1000 dilutions of Salmonella strains in parallel with the wild-type (N2) worms. The wild-type and dam-strains of Salmonella were diluted 1:100 and 1:1000 in E. coli OP50 and fed to N2 and JK905 worms on solid medium. Our results demonstrated that the glp-1 gene in C. elegans is not involved in Salmonella virulence since the death rates of the N2 (data not shown) and the JK509 worms were identical through the different conditions. The TD<sub>50</sub> for JK509 fed on Salmonella: E. coli dilution of 1:100 was 6.6 ± 0.6 and 7.6 ± 0.6 for wild-type and dam-mutant Salmonella, respectively. For the Salmonella: E. coli dilution of 1:1000, the TD<sub>50</sub> was 7.6 ± 0.3 and 10.3 ± 0.3 for wild-type and dam-Salmonella, respectively (Fig. 3). This analysis demonstrates that a 10-fold dilution in wild-type Salmonella is required to obtain the degree of attenuation conferred by the dam gene deletion. This analysis also validates the glp-1 deletion mutant for liquid based assays.

3.3. Dead C. elegans exhibit increased fluorescence when stained with SYTOX Green

Another challenge in the use of liquid cultures was distinguishing live and dead worms. On solid medium, worms not responding to a light touch were considered dead. In order to address this problem in liquid culture, we employed the SYTOX Green nucleic acid stain (Molecular Probes), which penetrates cells with compromised plasma membranes, but will not cross the membrane of live cells. We demonstrated that this dye is selective for dead worms (Fig. 4(a)) and that fluorescence increases in a dose-dependant manner (Fig. 4(b)). This calibration curve allowed us to determine the emission units corresponding to a known number of dead worms.

---

Fig. 2. Death rate of N2 C. elegans exposed to WT Salmonella SL1344, and dam-Salmonella SV4392 for 5 h, then fed on E. coli OP50 for 1 week. Wild-type Salmonella (squares) shows an ability to proliferate and kill C. elegans with minimal exposure. The dam-Salmonella (triangles) shows reduced virulence but still significantly greater than E. coli OP50 (circles). Values shown are means of ±standard error of triplicates.

Fig. 3. Temperature sensitive C. elegans JK509 fed Salmonella: E. coli dilutions on solid medium. Both wild-type (1:100, circles; 1:1000, squares) and dam-mutant (1:100, triangles; 1:1000, diamonds) Salmonella strains are virulent in C. elegans, though the dam-strain shows 10-fold attenuation. Values shown are means of ±standard error of triplicates.
3.4. *S. enterica* serovar *Typhimurium* shows attenuated killing of *C. elegans* in liquid culture

A smaller difference is seen between the TD$_{50}$ of JK509 worms fed wild-type *Salmonella* and *dam*-mutant in liquid medium, 5.8 ± 0.7 and 6.5 ± 0.2 days, respectively (Fig. 5). These results are comparable to the percent of dead worms at 4 days on the standard plate-based killing assay, after which time the killing by the wild-type strain is accelerated (Fig. 1). However, the obstacles of maintaining a healthy culture of both worms and bacteria in the same liquid media which required shorter timepoints, and the incomplete attenuation of virulence in the absence of DNA methylation indicated that further optimization is required. Nevertheless, the general approach which makes use of the JK509 strain and the counting of dead worms in liquid medium via SYTOX Green, should be useful in developing a screen for DAM inhibitors using *C. elegans*.

Fig. 4. Validation of SYTOX Green and a standard calibration curve of dead *C. elegans* in liquid media. (a) Fluorescence of live JK509 worms alone, live JK509 worms with SYTOX green dye, heat killed JK509 worms with SYTOX green dye, and an empty well measured. (b) Dose-dependant behavior is seen. The percentage of dead *C. elegans* is directly correlated with the emission signal; this correlation was used to convert emission data into percent dead worms in the liquid assay (Fig. 5). Fluorescence was measured by excitation at 485 nm and emission at 595 nm.

![Graph showing validation of SYTOX Green and a standard calibration curve of dead C. elegans in liquid media.](Fig. 4)

![Graph showing fluorescence of live JK509 worms alone, live JK509 worms with SYTOX green dye, heat killed JK509 worms with SYTOX green dye, and an empty well measured.](Fig. 4a)

![Graph showing dose-dependant behavior is seen. The percentage of dead C. elegans is directly correlated with the emission signal; this correlation was used to convert emission data into percent dead worms in the liquid assay.](Fig. 4b)

Fig. 5. Killing of *C. elegans* JK509 in liquid media. *C. elegans* feeding on *dam*-Salmonella (squares) showed reduced killing compared to the wild-type (triangles). This attenuation is partial and the mutant is significantly more virulent than *E. coli* OP50 (circles). Values are means of ±standard errors of eight repeated experiments.
4. Conclusions

A solid media killing assay developed by Aballay et al. [5] provides the basis for studying bacterial virulence mechanisms in C. elegans. We adapted this assay to evaluate the degree of attenuated virulence in a dam-mutant Salmonella [15,16], a highly specialized vertebrate pathogen. The assay was dependent on the ingestion of either wild-type or dam-mutant Salmonella by C. elegans hosts and a measurable difference in bacterial virulence resulting in worm death. Although the dam-Salmonella strain was shown to be completely avirulent in the mouse disease model, the results of the NGM solid media studies suggest that while removing DAM activity significantly reduces Salmonella virulence, complete attenuation is not observed. The mutant strain continues to express virulence factors sufficient for disease in C. elegans but not the mammalian models. Thus, fundamental differences in host response between mice and worms are suggested by these results, perhaps due to differences in specific and non-specific defense systems [5].

Infection in a liquid environment may be more difficult due to the reduced ingestion of bacteria by C. elegans as a result of stress. Jones and Candido [28] described a cessation in nematode pharyngeal pumping in a high stress environment, leading to the inhibited ingestion of food. With the number of stress factors increased in a liquid environment such as oxygen tension and increased dauer pheromone concentrations, nematodes may ingest fewer bacteria. While the application of the liquid media killing assay to screen for DAM inhibitors using C. elegans will require further optimization due to the incomplete attenuation of the dam-mutant, our results support the use of the nematode to study host-pathogen interactions.

Acknowledgements

We thank C. Wood and members of the Rothman Lab for contributing the N2 strain and helpful discussions. We also thank the Caenorhabditis Genetics Center for the JK509 strain and Professor P. Casadesus for providing us with S. enterica serovar Typhimurium strains SL1344 (wild-type), and S. enterica serovar Typhimurium SV4392 (dam-). This work was supported by NSF MCB-9983125 to NOR.

References


